



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

BANKIEWICZ et al.

Confirmation No.: 9216

Serial No.: 09/887,854

Art Unit: 1632

Filing Date: June 21, 2001

Examiner: S.L. Chen

Title: METHODS OF TREATING CENTRAL NERVOUS  
SYSTEM DISORDERS USING VIRAL VECTORS

**DECLARATION PURSUANT TO 37 C.F.R. 1.131**

Commissioner for Patents  
PO Box 1450  
Alexandria, VA 22313-1450

Sir:

We, Krys Bankiewicz and Janet Cunningham hereby declare as follows:

1. We are the coinventors of the pending claims in the above-captioned patent application, U.S. serial number 09/887,854 (hereinafter "the Application").
2. We understand the U.S. Patent and Trademark Office has cited the following against the pending claims: (1) Leff et al., *"Towards a gene therapy for Parkinson's disease (PD): Intrastriatal injection of recombinant adeno-associated virus (rAAV) encoding human L-aromatic amino acid decarboxylase (hAADC) in 6-OHDA lesioned rats restores AADC activity to control levels"* from the 27<sup>th</sup> Annual Meeting of the Society for Neuroscience, Part 1, New Orleans, LA, October 25-30, 1997 ("Leff"); and (2) Mizuno et al., *Jpn. J. Cancer Res.* (January 1998) 89:76-80 ("Mizuno").
3. We submit this declaration to show that before October 1997, we had conceived of the invention claimed in the Application, namely administering a recombinant adeno-associated virus ("AAV") virion to the brain of a subject suffering from a CNS disorder to provide widespread distribution of the recombinant AAV virion and achieve a therapeutic effect. Attached as Exhibit A are certain notebook pages which establish this. The dates on these notebook pages have been redacted. However, all of the work described on these pages predates October, 1997 and was performed in the United States.

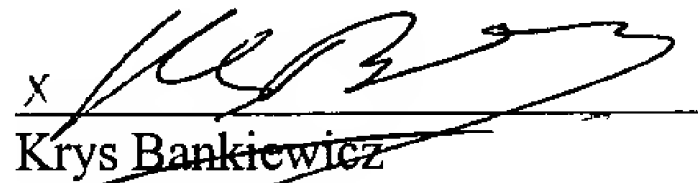
4. Prior to October, 1997, we delivered a recombinant AAV vector including the gene encoding herpes simplex thymidine kinase ("HSV-tk") to the brains of mice using a pump in order to provide widespread activity. See, notebook pages numbered 17 and 18 of Exhibit A. We initially used AAV-tk as proof of principle that AAV-mediated gene therapy could provide widespread distribution of AAV to the brain to achieve a therapeutic effect. In particular, at the time the experiments were performed, it was known that HSV-tk could be used to treat a variety of disorders, including cancer and viral diseases. See, Caruso and Klatzmann, *Proc. Natl. Acad. Sci.* (1992) 89:182-186 (attached as Exhibit B); Izquierdo et al., *Gene Ther.* (1996) 3:491-495 (abstract attached as Exhibit C); Freeman et al., *Semin. Oncol.* (1996) 23:31-45 (abstract attached as Exhibit D); Kruse et al., *Cancer Gene Ther.* (1997) 4:118-128 (abstract attached as Exhibit E); and the Mizuno reference cited by the Examiner. Thus, we conducted preliminary experiments with AAV-tk using a pump for delivery in order to determine whether we could provide widespread distribution of thymidine kinase in the brain to ultimately treat a CNS disorder. Following these preliminary experiments, we continued to work diligently with the HSV-tk gene, as well as with the gene encoding aromatic amino acid decarboxylase (AADC), another gene known to encode a protein useful in the treatment of a CNS disorder, Parkinson's disease. We indeed were successful at achieving widespread distribution of the AADC gene in primate brain, as detailed in the Application.

5. We believe that the results regarding widespread delivery of AADC can be extrapolated to the widespread delivery of various other proteins that were known to be deficient or missing in a variety of CNS disorders at the time the application was filed. Following the teachings in the application, a scientist skilled in the art of AAV-mediated gene therapy could readily prepare recombinant AAV virions including genes encoding such proteins, and deliver the virions to subjects with widespread distribution of virions to provide a therapeutic effect.

6. We declare that all statements made herein of our own knowledge are

true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date x 7/17/03

x   
Krys Bankiewicz

Date x 7/16/03

x   
Janet Cunningham



EXHIBIT A



TITLE

Project No. \_\_\_\_\_

Book No. 84

17

From Page No. \_\_\_\_\_

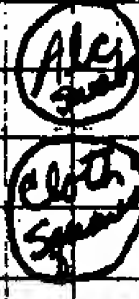
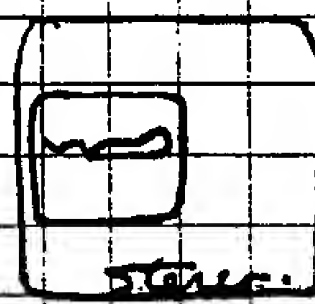
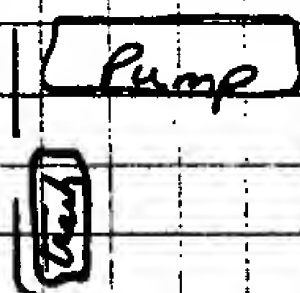
Date \_\_\_\_\_

← Animal Protocol # AS-97-02

\* Transduction of Normal Mouse Brain / AAV-tk

(Plan → 3 ages of Nod mice (#1 → AAV-lacZ, #2 → AAV-tk, #3 → Both)  
x 3 mice / gp  
9 mice total

(\* Several days early → Sign out tk + lacZ vectors and transfer from Avenir "stock" boxes to my own, at -80° (see p. 5))

- Lab Bench (get stuff); Viral Harvest aliquots { 12 µl tk, 12 µl lacZ, 6 µl each, mix (per animal)  
- Animal facility:Make up anesthetic → 140 µl Ket + 100 µl Xyl + 960 µl saline  
Setup board:Outside box  
gas bubble  
"Anesthetic" cap  
Tape  
Supply

Set pump → go thru "table" settings

+ select 25 µl glass Hamilton syringe, + select 2 µl Hamilton

Clamp down " " " w/ attached needle + tubing, all filled w/ saline!!!

- Rush plunger to 15 µl mark to dispense some; pull back 0.5-1.0 µl

load sample by pulling back to 25 µl  
\*\*\* SET PUSHER BLOCK gently AGAINST plunger!!!

(contd.)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Susan A. McLaughlin

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

Cunningham

Project No.

B ok No. 84

TITLE

Also →

+ One line (mouse) slide

→ BAD → All cells very red. But Nakai had fixed in para form. Not fixed, and tissue fixed same way as mouse, for Cy 3 control

From Page No.

FORMALIN-FIXED

NONE

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LAC-Z

TK

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LacZ

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24 OG-Anti-  
25 AMCA " " Almost none  
26 TR-Anti-  
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## EXHIBIT B





# Selective killing of CD4<sup>+</sup> cells harboring a human immunodeficiency virus-inducible suicide gene prevents viral spread in an infected cell population

MANUEL CARUSO AND DAVID KLATZMANN\*

Laboratoire de Biologie et Génétique des Pathologies Immunitaires, Centre National de la Recherche Scientifique, URA D1463, Hôpital de la Pitié-Salpêtrière, 83, Boulevard de l'hôpital, 75651 Paris Cedex 13, France

Communicated by Richard Axel, September 20, 1991

**ABSTRACT** We have stably expressed in CD4<sup>+</sup> lymphoid cells the herpes simplex virus type 1 thymidine kinase (HSV1-TK) gene under the control of the human immunodeficiency virus (HIV) promoter and transactivation response element sequences. Upon HIV infection these regulatory sequences were transactivated, switching on high-level expression of HSV1-TK. This in turn caused the death of HIV-infected cells when they were cultured in the presence of acyclovir, a nucleoside analog that becomes toxic after phosphorylation by HSV1-TK. The elimination of HIV-infected cells resulted in the arrest of HIV spreading in the culture. Complete protection of HSV1-TK-expressing cells was obtained using acyclovir concentrations that are commonly detected in the plasma of patients treated for HSV1 infection. Thus, expression of this DNA construct generates a pool of CD4<sup>+</sup> booby-trapped cells that, as a population, are resistant to HIV infection. Our data provide a rationale for the use of suicide genes in the design of gene therapy of HIV infection.

Human immunodeficiency virus (HIV)-induced diseases arise as the result of a persistent viral infection that spreads among CD4<sup>+</sup> lymphocytes, monocytic cells, or dendritic cells (1). All of these mature cells are believed to be derived from a common bone marrow progenitor. This unique origin makes it possible to design a therapeutic approach for HIV infection based upon the introduction into hematopoietic stem cells of genetic information that will inhibit HIV replication and/or spread. Such antiviral protection has been termed intracellular immunization (2). Different experimental approaches toward intracellular immunization against HIV have been designed. Initial studies utilized the expression of mutant HIV regulatory or structural proteins that exercised a trans-dominant negative effect on HIV replication (3, 4). Later, intracellular immunization approaches based on the expression of RNA were attempted. They involved expression of specific antisense RNA molecules (5) or of competing HIV promoter and transactivation response element sequences (6). For effective blocking of HIV replication all of these approaches require the constitutive high-level expression of either protein or RNA, which may explain why complete inhibition of HIV replication was not achieved. The HIV-induced expression of an interferon  $\alpha_2$  gene has also been assayed and shown to result in an efficient protection against HIV spread during 2-week-long cultures (7). However, this elegant approach would not eliminate HIV-infected cells. Consequently, to obtain efficient protection against HIV spread, we reasoned that it might be easier to sacrifice HIV-infected cells rather than to block viral replication.

The characteristics of HIV replication make it suitable for obtaining a rapid HIV-dependent suicide of infected cells.

First, the HIV promoter is inefficient in the absence of the HIV-encoded regulatory protein TAT (8–10). Second, there is a temporal program of mRNA accumulation during the HIV replication cycle. In the initial phase, multiply spliced mRNAs encoding HIV regulatory proteins, including TAT, are produced while the mRNAs encoding HIV structural proteins are not significantly expressed until 24 hr after viral infection, at which time virus production becomes apparent (11). Therefore, a toxic gene placed under the control of the HIV regulatory sequences will be expressed at a basal low level in noninfected cells and would be switched on in the initial phase of HIV replication, before the initiation of HIV structural protein synthesis. This should result in cell death before the release of newly synthesized viral particles and thus prevent viral spread.

Since the HIV promoter is not completely silent in the absence of TAT, the toxin used should exert its effect in a dose-dependent manner compatible with cell viability at a low level of expression. These are the characteristics of the herpes simplex virus type 1 thymidine kinase (HSV1-TK), which efficiently phosphorylates nucleoside analogs such as acyclovir (ACV) into monophosphorylated molecules. These in turn are subsequently converted into nucleotide triphosphates that can be incorporated into elongating DNA by cellular polymerases, causing chain termination and eventually cell death (12–14). This phenomenon can be achieved in mammalian cells in the absence of any other HSV1 protein. It has been used successfully in transgenic animals to conditionally deplete cell populations expressing a HSV1-TK transgene, a process termed “thymidine kinase obliteration” (15).

In this study we have stably expressed HSV1-TK, under the control of the HIV-1 long terminal repeat (LTR), in CD4<sup>+</sup> lymphoid cells. In the presence of ACV, we observed a specific and dose-dependent inhibition of HIV replication in these cells. With 10  $\mu$ M ACV, the cell population could be maintained free of any detectable HIV infection. We conclude that, as a population, cells harboring a HIV-inducible suicide gene are protected against HIV infection.

## MATERIALS AND METHODS

**Plasmids.** All subcloning was performed using standard cloning techniques (16). We first constructed the pLTR plasmid by removing the *Sal*I/*Xba*I env insert from pIIIEnv (3–1) (17); this was followed by Klenow blunting and religation. To construct pLTR-TK we first cloned the 2.7-kilobase (kb) *Bgl*II/*Bam*HI HSV1-TK insert from pFG5 (18) into the *Bam*HI site of pGEM3Z (Promega). The *Hind*III/*Pvu*II HSV1-TK insert obtained from this plasmid was then cloned

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Abbreviations: HIV, human immunodeficiency virus; HSV1-TK, herpes simplex virus type 1 thymidine kinase; ACV, acyclovir; LTR, long terminal repeat; TCID, tissue culture infectious dose of HIV.  
\*To whom reprint requests should be addressed.



in the *HindIII/Sma I* pLTR cloning sites, generating pLTR-TK. To obtain stable transformants we further fused the 2.8-kb *Xho I/BamHI* insert from pLTR together with a *Sal I/BamHI* fragment from pHYG (19), which codes for hygromycin resistance.

**Cell Culture and Transfection.** HUT-78 cells were maintained in RPMI medium supplemented with 10% fetal calf serum (Flow Laboratories). For transfection, cells were rinsed with ice-cold phosphate-buffered saline (PBS), pelleted by centrifugation, rinsed again, and then resuspended in RPMI medium at a density of  $4 \times 10^6$  cells per ml. They were kept on ice for 10 min and 0.8 ml of the cell suspension was added to a prechilled electroporation cuvette together with 20  $\mu$ g of plasmid. Two consecutive electric pulses (2.5 kV, 0.25  $\mu$ F and 0.3 kV, 960  $\mu$ F) were delivered to the cells using a Bio-Rad electroporator. Cells were allowed to recover on ice for 10 min, resuspended in 50 ml of culture medium, and distributed in the 48 wells of a multiwell plate (Falcon). After 48 hr, 500  $\mu$ g of hygromycin per ml was added. Half of the volume of the medium was gently removed every 3–4 days and cells were cultured in the selection medium until clones appeared. Six clones were obtained and analyzed by Southern blot analysis.

**Detection of HSV1-TK Gene and mRNA.** For Southern blot analysis, 15  $\mu$ g of genomic DNA digested with *EcoRI* was hybridized with a  $^{32}$ P-labeled HSV1-TK insert obtained from a *HindIII* digestion of pLTR-TK. For the determination of HSV1-TK copy number, we compared the hybridization signal obtained with genomic DNA to the one of serial dilutions of the pLTR-TK plasmid DNA representing the equivalent of 1, 5, and 10 copies per cell. For the detection of HSV1-TK mRNA, total RNA was extracted by the guanidium thiocyanate procedure. Serial RNA dilutions were slotted on a nylon membrane and hybridized with the  $^{32}$ P-labeled HSV1-TK probe. Copy numbers were estimated by comparison with the hybridization signal of  $10^6$ ,  $10^7$ , and  $10^8$  copies of an *in vitro* transcribed HSV1-TK insert (data not shown).

**Cell Proliferation and Viability Assays.** The effect of ACV (Zovirax; Burroughs Wellcome) on DNA replication was determined by a [ $^3$ H]thymidine incorporation proliferation assay. Cells ( $10^4$ ) were cultured in medium containing different concentrations of ACV. After 60 hr of culture, 1  $\mu$ Ci of [ $^3$ H]thymidine (1 Ci = 37 GBq) was added for another 7 hr of culture. [ $^3$ H]Thymidine incorporated in cellular DNA was measured after cell precipitation on Whatman paper using a Skatron apparatus. Cell viability was assayed by counting viable cells by trypan blue exclusion after 72 hr of culture with ACV.

**Virus and Viral Infection Procedures.** We used the LAV-LAI strain of HIV-1 (Diagnostics Pasteur, Marnes la Coquette, France). Cells ( $2 \times 10^5$ ) were infected in a 100- $\mu$ l final volume for 3 hr at 37°C with 100 tissue culture infectious doses of HIV (TCID). The cells were then washed once and resuspended in medium with different ACV concentrations. Cells were cultured in 24-well plates. Every 2 or 3 days, half of the cells were discarded, a sample of supernatant was withdrawn for the analysis of viral antigens, and fresh medium was added. For the detection of HIV-1 antigens we used the ELAVIA AgI Kit (Diagnostics Pasteur).

For the pLTR-TK transactivation assay,  $10^7$  HUT-78 or HUT-TK cells were infected with  $2 \times 10^6$  TCID for 6 hr at 37°C. Cells were then washed and cultured for another 22 hr. At that time total RNA was extracted and cytospin preparations were prepared. Serial dilutions of RNA from infected and noninfected cells were analyzed as described. Cytospin preparations were analyzed by immunohistochemistry using anti-p25 and anti-nef monoclonal antibodies as described (20).

## RESULTS

**Expression in CD4<sup>+</sup> Lymphoid Cells of a HIV-LTR-Driven HSV1-TK Gene.** We constructed a plasmid in which the HSV1-TK gene is under the control of the HIV-1 5' LTR (pLTR-TK). This plasmid also expresses the hygromycin-resistance gene driven by the weak TK promoter. pLTR-TK was electroporated into HUT-78 CD4<sup>+</sup> lymphoid cells, and stable transformants (HUT-TK cells) were selected with hygromycin. We first verified that these cells were expressing normal levels of CD4 (data not shown) and then analyzed their susceptibility to HIV infection. In 10 independent experiments, the kinetics of HIV replication, the amount of HIV antigens produced in culture, and viral cytopathic effects were similar in HUT-TK and HUT-78 cells. Southern blot analysis of HUT-TK genomic DNA with a HSV1-TK probe revealed the presence of one copy of the HSV1-TK gene per cell. No hybridization could be detected with HUT-78 genomic DNA (data not shown). Finally, no HSV1-TK mRNA could be detected in HUT-78 cells, whereas a signal corresponding to one to four copies per cell could be observed in HUT-TK cells (Fig. 1A).

The HIV-LTR of HSV1-TK should be transactivated by TAT during HIV replication. We thus analyzed the HSV1-TK mRNA levels during a single round of HIV replication in HUT-TK cells infected with high titers of virus. Cells ( $10^7$ ) were infected with  $2 \times 10^6$  TCID. Total RNA was extracted and cytospin preparations were prepared 28 hr after

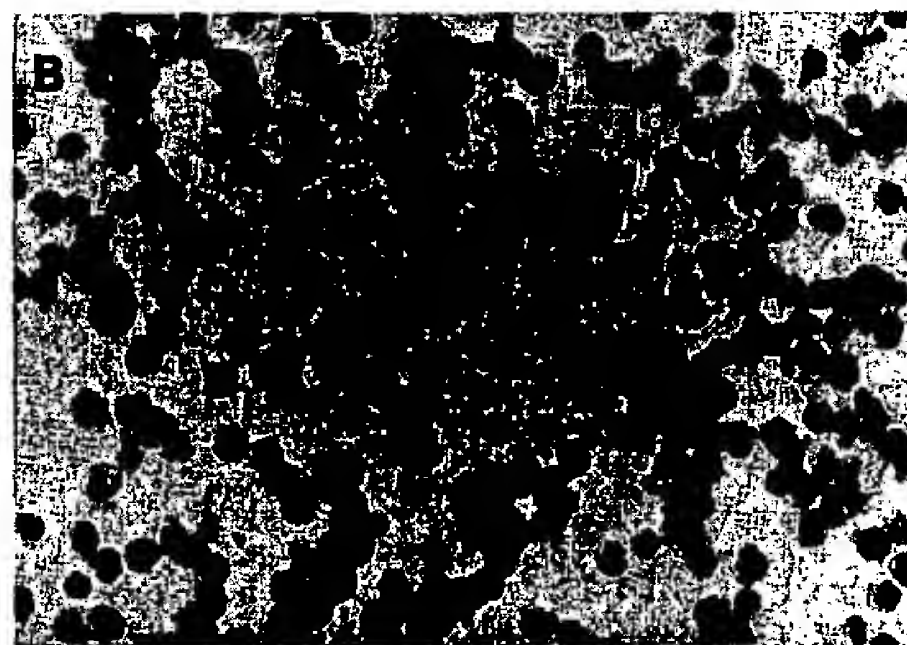
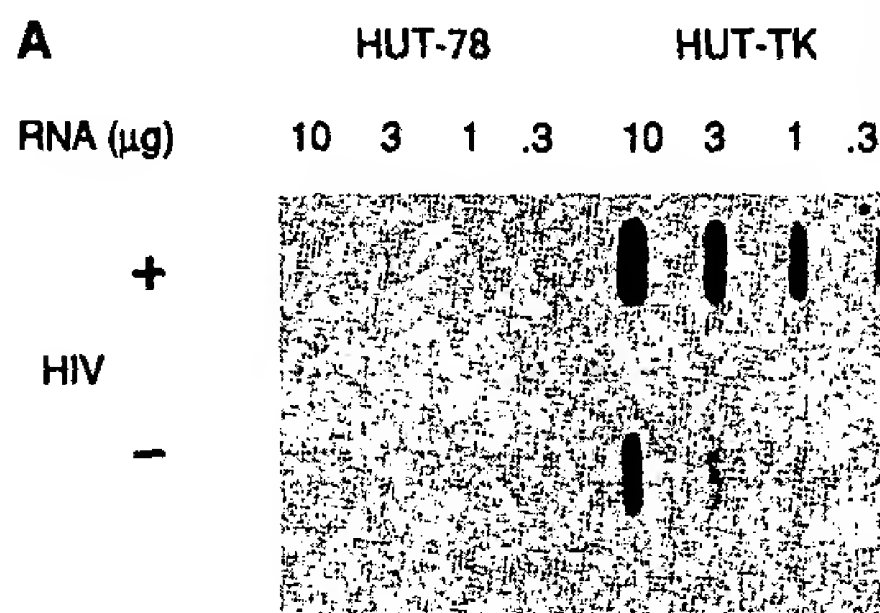


FIG. 1. pLTR-TK transactivation during HIV replication. HUT-78 and HUT-TK cells were infected with HIV for 28 hr and analyzed at this time for the expression of HSV1-TK mRNA and HIV antigens. (A) Serial dilutions of total RNA were slotted and hybridized with a HSV1-TK insert. (B) Immunoperoxidase staining of HUT-TK cells with an anti-p25 monoclonal antibody. Cells with a dark cytoplasm are expressing HIV-p25 and represent  $\approx 10\%$  of the cell population. ( $\times 100$ .)

infection. HIV replication in HUT-TK cells resulted in an  $\approx 10$ -fold increase in the level of HSV1-TK mRNA compared to the basal level observed in noninfected cells (Fig. 1A). As expected, no hybridization could be seen in HIV-infected HUT-78 cells. The cytospin preparations made at the time of RNA extraction were analyzed by immunohistochemistry with anti-HIV monoclonal antibodies (20). They revealed that  $\approx 10\%$  of the cells were infected, in agreement with the ratio of  $2 \times 10^6$  TCID per  $10^7$  cells that was used for this experiment (Fig. 1B). Thus, in each individual infected cell the transactivation of the HIV-LTR resulted in an average 100-fold increase in HSV1-TK expression. Further analysis of the HSV1-TK mRNA in an RNase protection assay demonstrated that these transcripts were correctly initiated at the HIV-LTR start site (data not shown). Similar results were obtained in two independent experiments. They demonstrate that HUT-TK cells have a low basal expression of the HSV1-TK gene that can be switched on during HIV replication.

We next examined the sensitivity of HUT-78 and HUT-TK cells to ACV. We assessed DNA replication by the incorporation of [ $^3$ H]thymidine and measured cell viability by trypan blue exclusion. No decrease in DNA replication could be detected in HUT-78 cells at ACV concentrations lower than  $10 \mu\text{M}$ , and the 50% inhibitory concentration ( $\text{IC}_{50}$ ) was  $\approx 300 \mu\text{M}$ . The sensitivity of HUT-TK cells to ACV was 10 times higher. No decrease in DNA replication was observed at ACV concentrations lower than  $1 \mu\text{M}$ , and the  $\text{IC}_{50}$  was  $30 \mu\text{M}$  (Fig. 2A). Our results are in good agreement with those

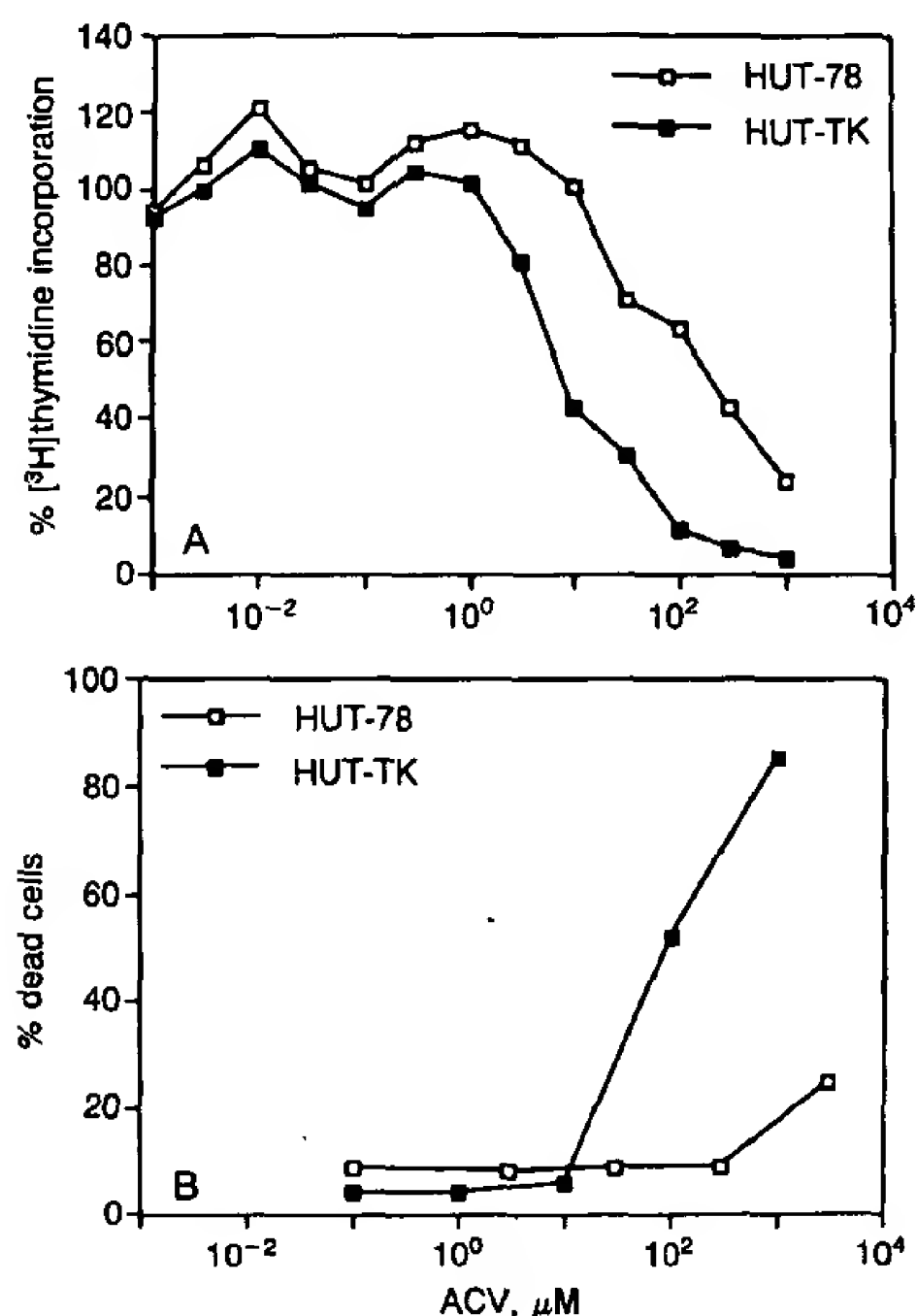


FIG. 2. Effect of ACV on DNA replication and cell death. HUT-78 parental and HUT-TK cells were cultured in the presence of increasing concentrations of ACV. After 6 days in culture, the effect of ACV on DNA replication and cell death was assessed by [ $^3$ H]thymidine incorporation and direct counting in the presence of trypan blue, respectively. (A) Effect of ACV on DNA replication. Results are expressed as percentage values obtained with cells cultured in the absence of ACV. (B) Effect of ACV on cell death.

obtained by Borrelli *et al.* (15), who also showed that at higher concentrations ACV becomes toxic for HSV1-TK-expressing mammalian cells. We observed that significant cell death occurs with concentrations of ACV over 100 and  $3000 \mu\text{M}$  for HUT-TK and HUT-78 cells, respectively (Fig. 2B). The moderate increase in sensitivity of HUT-TK cells to ACV indicates that these cells, which express low levels of HSV1-TK mRNA, are synthesizing a functional HSV1-TK.

**Effect of ACV on HIV Infection of Parental and HSV1-TK-Expressing Cells.** The dramatic increase of HSV1-TK expression in HIV-infected HUT-TK cells should increase their susceptibility to ACV. It should therefore be possible to find a concentration of ACV selectively toxic for HIV-infected cells. To test this hypothesis, HUT-78 and HUT-TK cells were infected with HIV and cultured in the presence of increasing concentrations of ACV. For HUT-TK cells, we used ACV concentrations ranging from 0.3 to  $10 \mu\text{M}$ . As shown in Fig. 3, we observed similar kinetics of HIV replication in HUT-TK cell grown in the presence of 0.3 or  $1 \mu\text{M}$  ACV, with the first appearance of HIV antigens on day 7. At  $3 \mu\text{M}$  ACV, the first detection of HIV antigens was delayed by 1 week and, at  $10 \mu\text{M}$  ACV, HIV infection was completely inhibited during the 17-day culture period (Fig. 3B). Identical results were obtained in three independent experiments. In these first two experiments we cultured HUT-78 cells in the presence of the same ACV concentrations used for HUT-TK cells. For all of these concentrations, the kinetics and extent of HIV replication were identical in the presence or the absence of ACV. In the third experiment we grew HUT-78 cells in the presence of ACV concentrations ranging from 10 to  $300 \mu\text{M}$ . As shown in Fig. 3, for all of the ACV concentrations used, HIV replication was first detected on day 7 in HUT-78 cells. At  $10 \mu\text{M}$  ACV, no significant modification in HIV replication could be observed, and at  $300 \mu\text{M}$  ACV, only a moderate decrease in the amount of HIV antigens was detected in the culture (Fig. 3A).

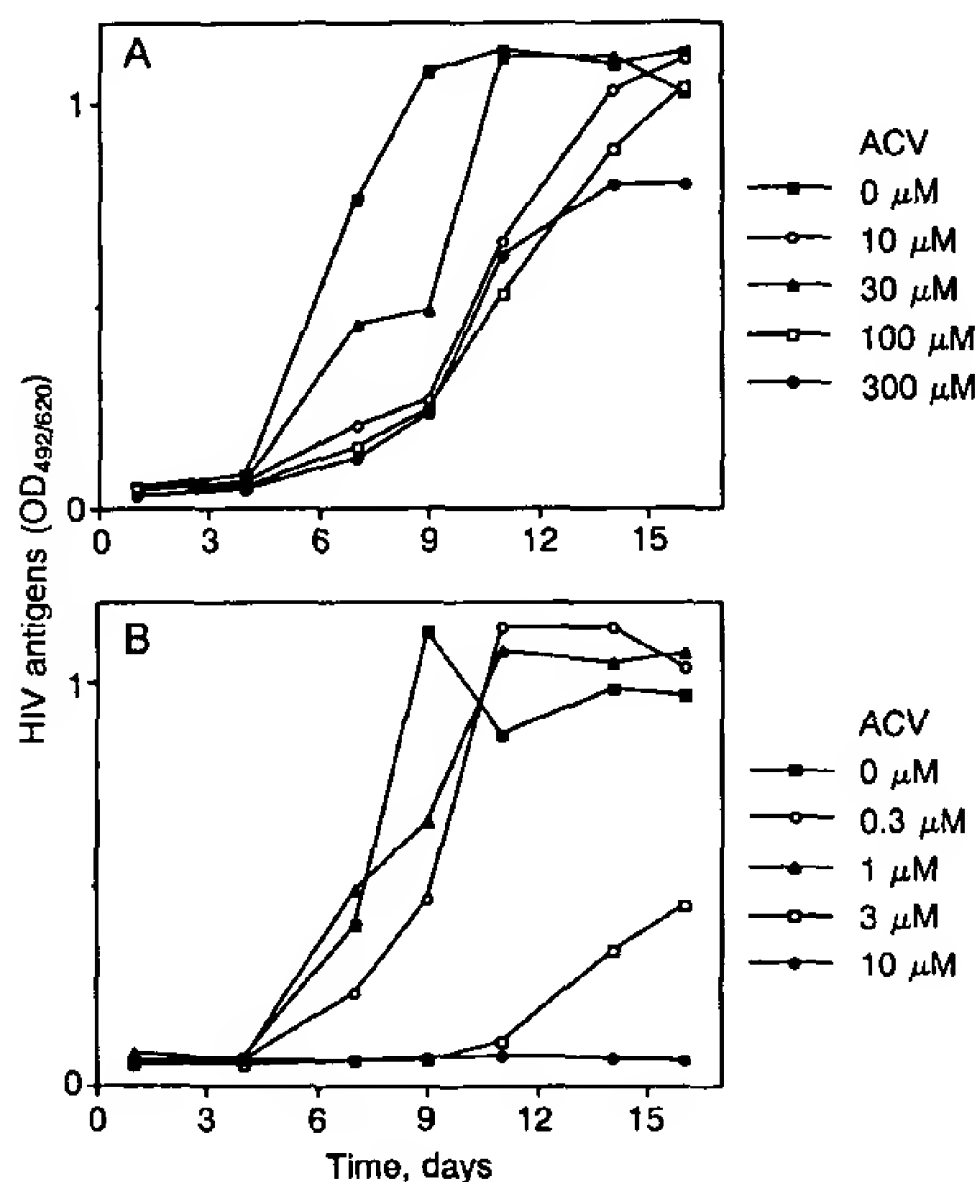


FIG. 3. Effect of ACV on HIV infection. HUT-78 (A) or HUT-TK (B) cells were infected with HIV and cultured in the presence of ACV. HUT-78 cells were cultured with ACV concentrations 30-fold higher than those used for HUT-TK cells. Every 3–4 days, HIV antigens were measured in the culture supernatant by ELISA.

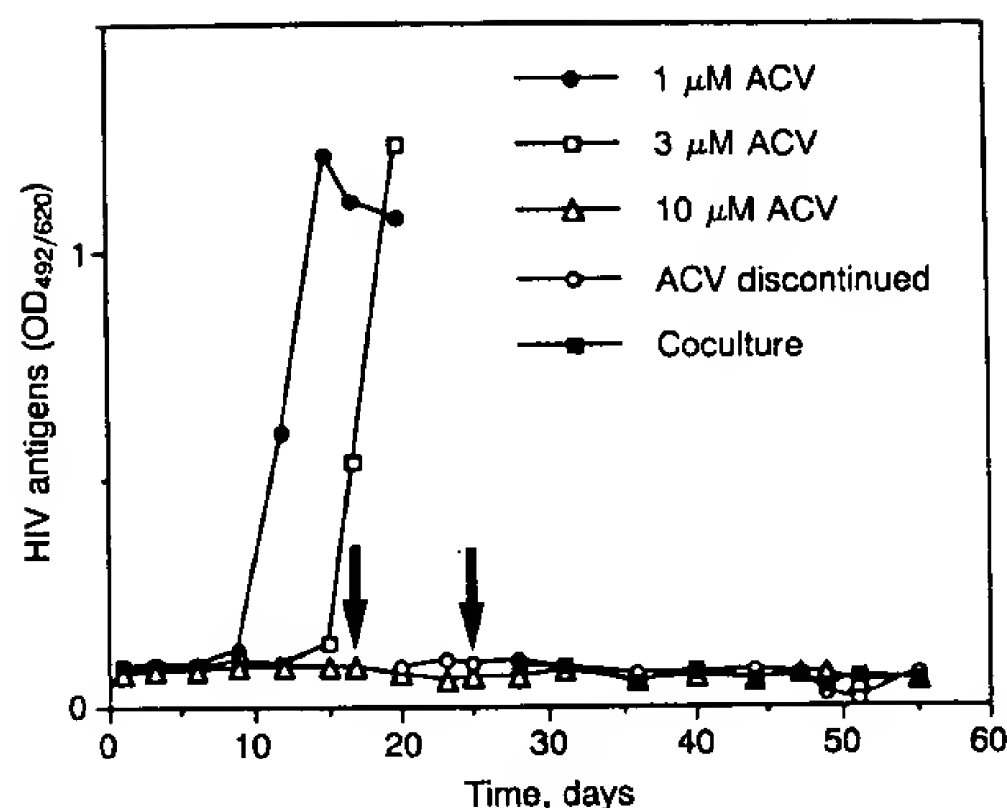


FIG. 4. Long-term effect of ACV on HIV infection of HUT-TK cells. HUT-TK cells were cultured in the presence of various ACV concentrations for 17 days. At this time point ACV was discontinued in half of the 10  $\mu$ M ACV-treated culture (left arrow, initiation of the culture without ACV). On day 25, half of the remaining ACV-treated cells grown with 10  $\mu$ M ACV were cocultured with parental HUT-78 cells (right arrow, initiation of the coculture). Results represent the measurement of HIV antigens in culture supernatants.

**Long-Term Effect of ACV on HIV Infection of HSV1-TK-Expressing Cells.** The failure to detect HIV replication in HUT-TK cells treated with 10  $\mu$ M ACV might be due to the elimination of infected cells. Alternatively, there could be a low level of HIV replication, below the sensitivity of the HIV antigen detection assay. In such a case, cocultivation with HUT-78 cells should permit these HIV particles to spread and to become detectable in these ACV-resistant cells. Furthermore, it is possible that HIV remained latent in ACV-treated HUT-TK cells, whereby discontinuation of the treatment would lead to a rapid reappearance of HIV replication. To test these different hypotheses, HUT-TK cells were infected and first grown in the presence of different doses of ACV. As previously shown, 3  $\mu$ M ACV delayed the appearance of HIV replication by 6 days, whereas at 10  $\mu$ M, HUT-TK cells were fully protected from HIV spread during the initial 17 days of the culture period. At this time point, we divided the culture so that half of the cells were maintained in the presence of 10  $\mu$ M ACV and the rest were grown in the absence of ACV. On day 25 the cells that were maintained in the presence of 10  $\mu$ M ACV were further divided into two cultures, one being cocultured with parental HUT-78 cells. No viral replication could be detected in any of these cultures for another 2-month period (Fig. 4). It is noteworthy that HUT-TK cells initially infected with HIV and grown in 10  $\mu$ M ACV could be cultured indefinitely without any toxic effects. Altogether, these results indicate that HUT-TK cells, as a population, were fully protected from HIV infection.

## DISCUSSION

We sought to design a genetic approach to the treatment of HIV infection based on the switching-on of a TAT-inducible suicide gene. Ideally, the suicide gene should encode an efficient and rapidly lethal toxin. These are the characteristics of the diphtheria toxin, which kills cells at an extremely low concentration (21). However, we were not able to generate viable transformants that stably expressed this toxin under the control of the HIV promoter, even after deletion of its enhancer elements (data not shown). We therefore used a toxin with a dose-dependent effect—namely, HSV1-TK. This enzyme, which by itself is not toxic for mammalian cells,

becomes toxic in the presence of specific nucleoside analogs. This toxicity is dependent on the amount of HSV1-TK and the dose of the nucleoside analog used. This dual dose-dependent effect can be clearly seen with an inducible expression vector for HSV1-TK (15). Rat fibroblastic cells cotransfected with HSV1-TK driven by a mouse mammary tumor virus promoter together with a glucocorticoid receptor expression vector were analyzed for their sensitivity to ACV before and after treatment with dexamethasone (DMS). Without DMS induction, high concentrations of ACV were necessary to kill cells expressing low levels of HSV1-TK. After DMS induction, which in this system increases the level of TK expression by 50-fold, cell death could be achieved at lower concentrations of ACV (15). Our system works on a similar principle since we have shown that in HUT-TK cells the HIV-LTR has a low-basal expression that is greatly increased by TAT.

Finally, HIV replication requires cell activation, which, in lymphoid cells, usually results in cell division (22, 23). In this regard, HSV1-TK-dependent ACV toxicity is mediated through the inhibition of DNA replication and thus also requires cell division. Therefore, HSV1-TK has the biological properties required to be used as a HIV-inducible suicide gene. We tested its efficacy by comparing the effect of ACV on HIV replication in parental HUT-78 and HUT-TK cells. We observed that ACV had no significant effect on HIV replication in HUT-78 cells, in agreement with previous observations (Peter Collins, Wellcome Research Laboratories, personal communication). In contrast, with 10  $\mu$ M ACV we obtained a complete protection of HSV1-TK-expressing cells throughout the 3-month culture period. This protection from HIV replication is clearly dependent on the expression of HSV1-TK and the concentration of ACV and should be due to the toxic effect of phosphorylated ACV for mammalian cells. Thus, the suicide of individual infected HUT-TK cells completely blocked the viral spread in the culture and resulted in the full protection of the cell population. In the context of a HIV-infected patient, whose chronic disease is the result of constant viral spreading, the presence of a population of LTR-TK harboring cells should reduce viral spread with the result that HIV infection might even abort.

As previously stated (4), the difficulties encountered in developing effective therapeutic agents against HIV infection justify the search for genetic approaches to the therapy of HIV infection. Any approach of this kind would require that HIV target cells of patients whose immune systems are reconstituted with engineered bone marrow precursors cells or mature lymphoid cells would be resistant to HIV infection and spread. Venkatesh *et al.* (24) have proposed the use of a recombinant adenovirus harboring a construct similar to ours to deliver HSV1-TK to HIV-infected cells *in vivo*. They showed that following infection, such a recombinant adenovirus could confer sensitivity to ACV onto HeLa cells constitutively expressing TAT. However the authors did not test their vector in CD4<sup>+</sup> cells and thus the effect of HSV1-TK on HIV replication could not be tested in this experimental system. We favor the idea that it would be better to generate a population of booby-trapped cells already protected from HIV spread. An elegant approach would be to use HIV pseudo-particles that would deliver the genetic information precisely to HIV target cells. However, the titers of such pseudo-viral particles are not yet sufficient for efficient gene transfer into hematopoietic cells (25, 26). The best system might be the use of murine amphotropic retroviral vectors and packaging cell lines (27, 28). New generations of such cell lines and vectors have been constructed with improved efficacy and safety (29, 30) and gene transfer in humans has already been performed using such systems (31). Thus, the means for carrying out TK obliteration *in vivo* do exist. It should be possible to test its efficacy in animals

that serve as models for HIV infection, such as monkeys or severe combined immunodeficiency mice whose immune systems are reconstituted with engineered human hematopoietic cells (32).

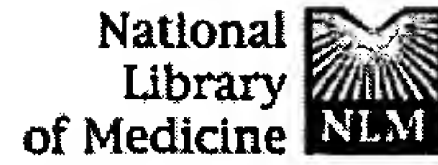
We are indebted to Patrick Salmon for setting up the experimental procedure for electroporating HUT-78 cells, Carlo Parravicini for help with immunochemistry, and Françoise Chapuis for helpful technical advice. We are grateful to Richard Axel, Marc Alizon, Olivier Danos, Jean-Michel Heard, Jean-Claude Gluckman, Benoît Salomon, and Michael Heiber for helpful discussion and critical reading of the manuscript. We also thank Virginie Blanchard for preparation of the manuscript. This work was supported by a grant from the Agence Nationale de Recherches sur le Sida. M.C. is a Fellow from Agence Nationale de Recherches sur le Sida.

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## Human malignant brain tumor response to herpes simplex thymidine kinase (HSVtk)/ganciclovir gene therapy.

Izquierdo M, Martin V, de Felipe P, Izquierdo JM, Perez-Higueras A, Cortes ML, Paz JF, Isla A, Blazquez MG.

Departamento de Biología Molecular/Centro de Biología Molecular Severo Ochoa, Facultad de Ciencias (modulo-C-X), Universidad Autónoma de Madrid, Spain.

Growing cells from human brain tumors have been treated in vitro and in vivo with murine therapeutic retroviral producer cells. The therapeutic retrovirus carried the potential suicide gene thymidine kinase (tk) from the herpes simplex virus (HSV). After a few days, in which a large proportion of the tumoral cells had the opportunity to acquire a copy of the retrovirus treatment with ganciclovir was initiated and considered responsible for considerable cell death both in vitro and in vivo. The in vivo experiments were performed in five adult patients who had failed standard therapy and were expected to survive only a few weeks.

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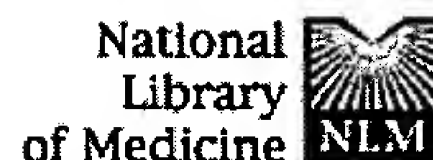
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1: Semin Oncol. 1996 Feb;23(1):31-45.

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## In situ use of suicide genes for cancer therapy.

Freeman SM, Whartenby KA, Freeman JL, Abboud CN, Marrogi AJ.

Department of Pathology, Tulane University Medical School, New Orleans, Louisiana, US

Gene therapy has now become a standard experimental approach for treating cancers that have failed conventional therapies. As the understanding of the molecular nature of carcinogenesis develops, new approaches are being taken to directly target tumor cells, thus bypassing the difficulties of killing cells that are resistant to chemotherapy and radiation. One emerging gene therapy approach has been through the genetic modification of tumor cells with a suicide gene such as the herpes simplex virus thymidine kinase gene (HSV-TK) and ganciclovir (GCV) therapy. Death of tumor cells modified with the HSV-TK gene leads to killing of unmodified in situ tumor cells in a phenomenon termed the "bystander effect." The basis both for this effect and other gene therapy trials underway for the treatment of cancer will be discussed.

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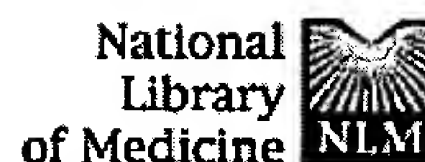
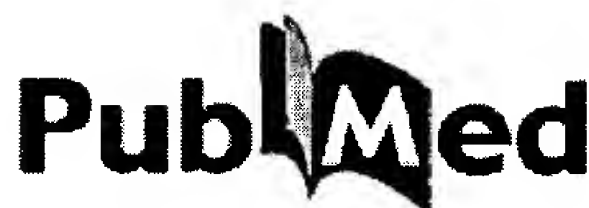
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1: Cancer Gene Ther. 1997 Mar-Apr;4(2):118-28.

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# **Purified herpes simplex thymidine kinase Retrovector particles. I. In vitro characterization, in situ transduction efficiency, and histopathological analyses of gene therapy-treated brain tumors.**

**Kruse CA, Roper MD, Kleinschmidt-DeMasters BK, Banuelos SJ, Smiley WR, Robbin JM, Burrows FJ.**

University of Colorado Health Services Center, Department of Immunology, Denver 80262, USA.

Replication-defective, highly purified retroviral vectors (Retrovector), at titers of 10(8) colony forming units/mL, were prepared that conferred either beta-galactosidase or herpes simplex thymidine kinase (HSV-TK) activity. 9L gliosarcoma cells, transduced efficiently in vitro, were highly sensitive to ganciclovir (GCV). The mean frequency of in situ transduction, measured by flow cytometry of single-cell tumor suspensions isolated from rat brains, was 3.2 +/- 0.6%; similar assessments were made by staining of beta-galactosidase or by immunohistochemistry with anti-HSV-TK. In vitro HSV-TK-transduced and G418-selected 9L-TK gliosarcoma tumors treated with GCV were eradicated in approximately 53% of the animals (10/19) at day 26, however, 89% (17/19) histologically showed < 1% tumor volume. Histologic evaluation at day 26 of animals with established 9L tumors treated with intralesional injection of HSV-TK vector followed by GCV treatment showed that 29% (4/14) had no tumor; 50% (7/14) had < 1% tumor volume. Regression of tumors proceeded over the time since the complete rate was increased at day 60. Neither HSV-TK vector particles nor GCV alone altered the histological profile of 9L tumors, but substantial numbers of CD4+ and CD8+ lymphocytes infiltrated the tumors of animals treated with both. In cured animals, the former tumor bed contained cell debris, immune cells, and fibroblasts and was without damage to adjacent brain. The efficacy of suicide gene therapy for rat gliosarcoma using highly purified virion vectors approaches that of packaging cell lines.

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